PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publication Number: **WO 90/15331**
G01N 33/68	**A1**	(43) International Publication Date: 13 December 1990 (13.12.90)

(21) International Application Number: PCT/US90/02698

(22) International Filing Date: 21 May 1990 (21.05.90)

(30) Priority data:
359,822   1 June 1989 (01.06.89)   US

(71) Applicant: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors: CARD, John, Patrick ; 1102 Foulk Road, Wilmington, DE 19803 (US). DAVIS, Leonard, George ; 3 Meteor Lane, Newark, DE 19711 (US). SIMAN, Robert, Gordon ; 2646 Bittersweet Drive, Wilmington, DE 19810 (US).

(74) Agents: CHRISTENBURY, Lynne, M. et al.; E.I. du Pont de Nemours and Company, Legal Department, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent)*, DK (European patent), ES (European patent), FR (European patent), GB (European patent), HU, IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

---

(54) Title: DIAGNOSTIC ASSAY FOR ALZHEIMER'S DISEASE

(57) Abstract

A method to assist in the diagnosis of Alzheimer's disease comprising detecting, in bodily fluids, two APP-related proteins, in soluble form, said proteins have an apparent molecular size of about 130 kDa and about 35 kDa, and each of said proteins shares at least one epitope with the C-terminus of APP corresponding substantially to amino acids 676-695 of APP as shown in Figure 1.

---

* See back of page

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

Title
DIAGNOSTIC ASSAY FOR ALZHEIMER'S DISEASE
Field of the Invention

This invention relates to an _in vitro_ diagnostic assay to assist in the diagnosis of Alzheimer's Disease and, more particularly, to an assay for detecting, in bodily fluids, unique soluble amyloid precursor protein-related proteins, fragments thereof, which share at least one epitope with the C-terminus of APP.

Background of the Invention

Alzheimer's Disease is a form of dementia marked by progressive intellectual deterioration without focal, motor or sensory signs. It is an insidious, progressive, debilitating disease of unknown etiology afflicting almost two million Americans, most of them elderly.

Definitive diagnosis of the disease is often difficult since many other disorders and neurological deficits are also accompanied by cognitive loss, and histological examination of brain biopsies is an unacceptable procedure in the majority of cases. Thus, only a very small percentage of the total population of Alzheimer's patients is diagnosed early, and those individuals successfully identified are often well advanced in the disease process. Moreover, multiple psychological and biological tests need to be performed to eliminate other non-Alzheimer's related causes of dementia (NIH Publication No. 84-2251, 1984). Consequently, appropriate treatments are difficult to plan. Determining whether one of the causes of dementia is Alzheimer's requires a tool which unequivocally detects Alzheimer's Disease or, at least,

eliminates most of the non-Alzheimer's related causes
of dementia.

Although the etiology of the disease is unknown,
researchers have identified three histopathological
structures which are present in the brains of
Alzheimer's patients: neurofibrillary tangles,
neuritic (senile) plaques, and cerebrovasculature
plaques. Neurofibrillary tangles (NT) are
intracellular accumulations of fibrous material in the
cell bodies of affected neurons, mainly in the
hippocampus, amygdala and neocortex. Neuritic and
cerebrovascular plaques are found in highest
concentration in the hippocampus and neocortex and
result from a pathological deposition of amyloid
precursor protein(s) (APP) or fragments thereof in
these regions. It should be noted that the term
amyloid, as used in a neuropathological context, refers
to the deposition of APP and its fragments into
plaques. This differs from the usage of the term in
the general, histopathological context. Specifically,
when applied to neuropathological plaques, the term
most commonly refers to the A4 amino acid fragment of
APP. In standard histopathological uses the term
anyloid refers to a refractive, insoluble, noncellular
material.

Formation of plaques is now known to be one of the
earliest events in the progression of the disease
(Terry et al., J. Neuropathol. Exp. Neurol. 46:262-268
(1987); Wisniewski et al., Banbury Report, Davies and
Finch, eds. (Cold Spring Harbor Laboratory, NY, pp.
1-26 (1988))). The clinical significance of plaque
formation has been highlighted by quantitative studies
showing a significant correlation between the numbers
of neuritic plaques and neurofibrillary tangles and the
clinical severity of dementia (Roth et al., Nature, pp.

109-110, Vol. 50 (1966) and Blessed et al., Br. J.
Psych., pp. 797-805, Vol. 114 (1968)), i.e., the most
serious cognitive deficits are correlated with the
largest number of plaques. Correlation of cognitive
deficits with plaque deposition has led to a concerted
effort in recent years to define the plaque
constituents and the mechanism(s) of plaque generation.

It has been demonstrated that an insoluble material
known as β-amyloid (Selkoe et al. Science 115:1243-1245
(1982)); (Glenner and Wong Biochem. Biophys. Res.
Commun. 122:885-890 (1984); Wong et al. Proc. Natl.
Acad. Sci. USA 82:8729-8732 (1985)), β-protein, or A4
(Masters et al. EMBO J. 4:2757 (1985)), which was
isolated from the birefringent amyloid core of plaques,
is the principle pathological component of Alzheimer's
Disease.

In 1984, Glenner and Wong (Biochem. Biophys. Res.
Comm. 122:1131-1135 (1984)) isolated and determined the
amino acid sequence of a peptidergic component of the
cerebrovascular amyloid plaques. Additional work with
peptides isolated from neuritic plaques from
Alzheimer's and Down's syndrome patients was done
because older Down's syndrome patients also develop
progressive dementia quite similar to the dementia
associated with Alzheimer's disease (Ellis et al.
Neurology 24:101-106 (1974), Wong et al., Proc. Nat.
Acad. Sci., USA, pp. 8729-8732, Vol. 82 (1985)). These
additionally isolated peptides were characterized and
all were found to contain an amino acid sequence which
was similar or identical to that originally isolated
and reported by Glenner.

The amino acid sequence determined by Glenner and
Wong has enabled researchers to use standard
recombinant DNA procedures to isolate cDNA clones that
encode the mRNA for the precursor protein of this

β-amyloid peptide (Kang et al. Nature 733 (1987); Tanzi
et al. Science 235:877 (1987)); Robakis et al. Proc.
Natl. Acad. Sci. USA 84:4190 (1987)). Additional
research has demonstrated that alternate forms of the
precursor mRNA and protein exist (Kitaguchi et al.
Nature, pp. 530-532, Vol. 331 (1988); Tanzi et al.
Nature 331:528-530 (1988); Ponte et al. Nature, pp.
525-527, Vol. 331 (1988)). Based on these cDNA
sequences, the amino acid sequences of the entire
protein molecule(s) can be predicted from the open
reading frame/coding region of the mRNA. Figure 1 sets
forth the predicted amino acid sequence for APP. It
was speculated that the protein would be membrane-
bound, possibly a receptor molecule (Kang et al. Nature
325:733 (1987)). Additional reports supported the
conclusion that the amyloid precursor protein (APP) is
a membrane localized protein (Zimmerman et al. EMBO J.
7:367-372 (1988); Allsop et al. Proc. Natl. Acad. Sci.,
pp. 2790-2794, Vol. 85 (1988)). More recently, an N-
terminal fragment has been reported in the soluble
fraction from tissue extracts (Palmert et al. cited in
Abraham and Potter Bio/Technology 7:147-153 (1989)).

It is also mentioned in Bio/Technology 7:147-153
that protein studies indicated APP is a membrane
glycoprotein which can be detected in two forms using
immunoblotting--a membrane-bound form which can be
stained with antibodies to the N-terminal and C-
terminal portions and a soluble form detected only with
N-terminal antibodies. It is further suggested that
the soluble form lacks the C-terminus, including the β-
protein. It is also mentioned that pulse chase
experiments showed that the soluble, extracellular form
is derived from the membrane-bound form, probably by
proteolytic cleavage. Results of protein studies are

presented in Table 1 appearing on page 150 of
Bio/Technology as discussed above.

McDonald et al., Alzheimer Disease and Associated
Disorders, page 186, Vol. 2, No. 3, 1988, have reported

5   the characterization and purification of a protein
present in the cerebrospinal fluid and serum of
individuals with Alzheimer's Disease using a monoclonal
antibody which reacts immunohistochemically with
amyloid deposits. Immunoreactive proteins of apparent

10   molecular size of 100 and 95 kDa were detected·using
this monoclonal antibody.

Selkoe et al., Proc. Natl. Acad. Sci. USA, pp.
7341-7345, Vol. 85 (October 1988), disclose the
detection of a group of ~~110 to 135 kDa membrane-bound

15   proteins in human brain, nonneural tissues and cultured
cells found to contain APP mRNAs. These proteins were
detected by immunoblot using antibodies specific to C-
terminal peptides of the predicted precursor protein
comprising either the 20 C-terminal amino acids

20   (numbers 676-695) or the 15 C-terminal amino acids
(numbers 681-695). No 35 kDa protein appears to have
been detected. Detection of a soluble form of
precursor proteins, sharing at least one epitope with
the C-terminus of APP, in cerebrospinal fluid or other

25   bodily fluids was not reported.

European Patent Application Publication No.
285,159, published on October 5, 1988, describes a
human amyloid related protein monoclonal antibody. It
is mentioned in column 9 at lines 13-24 that this

30   antibody is potentially useful to diagnose Alzheimer's
dementia. The amyloid-related protein recognized by
this antibody has a molecular size of about 42-45
kilodaltons.

European Patent Application Publication No.

35   274,826, published on July 20, 1988, describes the

generation of antibodies against recombinant
Alzheimer's amyloid protein or immunogenic peptides
thereof for cerebral fluid or serum diagnosis of
Alzheimer's Disease. There is no indication that one
5   soluble form of APP-related proteins sharing at least
one epitope with the C-terminus of APP could be
detected in bodily fluids.

Pardridge et al., Biochem. and Biophys. Res.
Commun., pp. 241-248, Vol. 145, No. 1 (May 29, 1987),
10   describes the development of a radioimmunoassay to
detect amyloid (A4) peptide or its precursor in human
serum or cerebrospinal fluid. A high molecular weight
immunoreactive substance was detected in human serum
and CSF samples. It is stated on page 245 that the
15   immunoreactive substance in serum or CSF is a high
molecular weight protein that is highly homologous or,
possibly, identical to the A4 peptide precursor, but
was not the amyloid peptide, per se. A synthetic
peptide corresponding to the first 28 amino acids of
20   the 43 amino acid A4 peptide coupled to bovine
thyroglobulin was used to generate the polyclonal
antiserum used to detect this protein.

Japanese Patent Application 62[1987]-267,297, which
was published on November 19, 1987, discloses a
25   monoclonal antibody which reacts against senile
plaques. It is mentioned on page 15 that it might be
useful in early diagnosis of senile dementia
Alzheimer's Type by detecting β-amyloid or its
precursor protein possibly present in serum or
30   cerebrospinal fluid.

Brief Description of the Drawings

Figure 1 depicts the approximately 695 amino acid
sequence deduced for APP. Numbering is according to
35   Kang group.

Summary of the Invention

A method to assist in the diagnosis of Alzheimer's disease comprising detecting, in bodily fluids, two APP-related proteins, in soluble form, having an apparent molecular size of about 130 kDa and about 35 kDa wherein each protein shares at least one epitope with the C-terminus of APP corresponding substantially to amino acids 676-695 as shown in Figure 1 (numbering according to Kang group) or a subsequence thereof.

Detailed Description of the Invention

The term Alzheimer's amyloid precursor protein (APP) refers to the approximately 695 amino acid sequence and the alternate forms which have been deduced as the putative sequence for the Alzheimer's amyloid precursor. Figure 1 depicts the putative amino acid sequence for the 695 APP form, numbering according to the Kang group.

The term APP-related proteins refers to APP, its alternate forms, cross-immunoreactive fragments thereof, and other proteins which cross-immunoreact with antisera directed against peptide sequences of the APP protein.

The term A4 peptide refers to the approximately 42-43 amino acid sequence identified by Masters et al. While the first twenty-eight amino acids (termed β-amyloid) was identified by Glenner et al., _Biochem. and Biophys. Res. Commun._, pages 885-890, Vol. 120, No. 3 (1984) and U.S. Patent 4,666,829 which issued on May 19, 1987.

The term "corresponding substantially" means functional equivalents with conservative substitutions, additions or deletions also fall within the scope of this invention.

Surprisingly and unexpectedly, unique soluble forms
of APP-related proteins, having apparent molecular
sizes of about 130 kDa and 35 kDa, respectively and
also sharing at least one epitope with the C-terminus
of APP corresponding substantially to amino acids
676-695 of APP as shown in Figure 1, were detected in
cerebrospinal fluid (CSF) of Alzheimer's samples
according to the procedure described below.

An approximately 130 kDa protein (hereinafter "130
kDa protein") was detected in soluble form in the CSF
of all the Alzheimer's samples which were analyzed (14
samples). In contrast, the 130 kDa protein was not
detected in 5 normal control samples. The 130 kDa
protein was detected in CSF of 5 of 12 degenerative
disease states (Parkinson's, Pick's, or Creutzfeldt-
Jakob's), and in 4 of 5 aged samples having an unclear
neurological status (liver cirrhosis, heart failure,
alcoholism/infarcts, pneumonia, or acute hypoxia). It
is believed that the 130 kDa protein is an APP-related
protein which shares at least one epitope with the C-
terminus of APP corresponding substantially to amino
acids 676-695 of APP as depicted in Figure 1 or a
subsequence thereof. Results are presented in Table 1.

An approximately 35 kDa protein (hereinafter "35
kDa protein") in soluble form was detected in the CSF
in ten Alzheimer's samples (the other four samples
could not be analyzed as they were not resolved on the
earlier gels and none of the samples are left).

The 35 kDa protein was not detected in the 5 normal
control samples. It was detected in 6 of 12
degenerative diseases (Parkinson's, Pick's or
Creutzfeldt-Jakob's), and in one of the aged samples
having unclear neurological status (liver cirrhosis,
heart failure, alcoholism/infarcts, pneumonia, or acute
hypoxia); the other four aged samples having unclear

neurological status were not analyzable for the reasons described above.

The 35 kDa protein also appears to be an APP-related protein which shares at least one epitope with the C-terminus of APP corresponding substantially to amino acids 676-695 of APP as depicted in Figure 1 or a subsequence thereof. Thus, it is believed that by detecting these unique soluble forms of APP-related proteins having apparent molecular sizes of about 130 kDa and about 35 kDa in bodily fluids and which share at least one epitope with the C-terminus of APP as described above, will reduce some of the testing for the non-Alzheimer's causes of dementia. Results are presented in Table 1 below.

It should be noted that some individuals who are diagnosed as having Parkinson's disease may also suffer from Alzheimer's Disease. However, the diagnosis is not modified to reflect that Alzheimer's Disease is also present (NIH Publication No. 84-2251 (1984)). Thus, some of the Parkinson's samples which tested positive for the APP-related 130 kDa and 35 kDa proteins, might also have Alzheimer's Disease.

Regarding the detection of the 130 kDa and 35 kDa proteins in CSF samples taken from individuals suffering from Creutzfeldt-Jakob's disease or Pick's disease, it is interesting to observe that individuals suffering from these diseases exhibit plaque-like deposits. In particular, Pick's disease is a rare form of presenile dementia with specific pathologic findings. Histologically, there is marked neuronal loss, particularly in the outer three layers of cortex of the frontal lobes and anterior temporal lobes. Clinically, this process is indistinguishable from Alzheimer's disease. Creutzfeldt-Jakob is a well defined clinical disease state which was formerly classified as presenile dementia of unknown etiology.

It is unclear to what extent amyloid is associated with
Creutzfeldt-Jakob disease.

These APP-related proteins were detected using an
immunoblot. It was important that the samples were
concentrated to a total protein concentration in the
range from about 1.0 mg/ml to about 3.5 mg/ml using
conventional techniques, such as ultrafiltration,
freeze drying, evaporation, reverse osmosis, etc. The
preferred concentration was about 2.5 mg/ml. Those
skilled in the art will appreciate that the protein
concentration desired can vary depending upon the
sensitivity of the reporter system and affinity of the
antibodies as well as the quality of the samples.
Thus, the desired concentration can vary depending upon
these factors.

In addition to cerebrospinal fluid, a variety of
other bodily fluids such as blood, plasma, serum, urine
and the like, can be used in the assay of this
invention.

Proteins in the samples were separated by SDS-
polyacrylamide gel electrophoresis on 5 to 15% linear
gradient gels as described below.

APP specific antibodies were generated against
various polypeptides corresponding to subsequences of
the APP using conventional techniques as is described
below. It is also within the scope of this invention
to use monoclonal antibodies or immunoreactive
fragments thereof to identify the presence of amyloid
precursor protein, fragments thereof, and abnormalities
in APP structure or physiological levels that might
exist in the disease state when compared to results
obtained from normal samples.

Monoclonal antibodies can be generated using the
immunogens described below as well as other immunogens

based on the APP sequence set forth in Figure 1 and
standard hybridoma technology such as that described in
U.S. Patent 4,196,265 and Kohler et al., Nature, pages
495-497 Vol. 256 (August 7, 1975).

5   For example, Balb/c x C57B16 mice can be immunized
with the polypeptides coupled to a carrier protein such
as keyhole limpet hemacyanin via the glutaraldehyde
method of A. Kagan et al., Methods of Hormone
Radioimmunoassay, pp 327-339 (2d Ed.) (1979). It is
10   also possible to immunize mice with uncoupled
polypeptides as was done and as described below.

          Three days after an intraperitoneal boost the
spleens of the appropriate immune mice are removed and
fused with a non-secretor myeloma cell. Spleen cell
15   suspensions are prepared in serumless DMEM-high glucose
medium and mixed with myeloma cells at a ratio of 4:1.
This cell mixture is centrifuged at 1200 g for 10
minutes at room temperature. After removal of the
supernatant, the cells are resuspended by gently
20   tapping the tube. The fusion procedure is initiated by
adding 1.0 ml of 45% w/v polyethylene glycol 3350
(Baker) at 37°C over a 30-second period.

          The cells are occasionally mixed with a pipette tip
for 90 seconds and 5 ml of serumless DMEM-high glucose
25   medium is added over a 3-minute period. This is
followed by the addition of 14 ml of DMEM-high glucose
supplemented with 10% fetal calf serum, L-glutamine,
hypoxanthine, aminopterin and thymidine (referred to as
HAT medium). The HAT medium is added over a 1-minute
30   period.

          Appropriate volumes of HAT medium are added to
cells and then the cells are centrifuged at 800 x g for
7 minutes at room temperature. Supernatants are
aspirated and the cell pellet is disrupted with 10 ml
35   of HAT medium. Peritoneal cells from Balb/c x C57B1/6

are added and the final volume adjusted so that two
hundred thousand spleen cells are dispensed to each
well. Approximately 14 days later, tissue culture
supernatants from wells containing hybridoma colonies

5   are tested by ELISA for the desired reactivity with
peptides conjugated to other carrier proteins.

It was found that the sensitivity of detection by
immunoblot increased about three-fold by incubating the
APP-specific-antibody-APP-related proteins complex

10   with a detector system which comprised two detector
reagents at two different dilutions after concentrating
protein from the samples to a desired level. Following
incubation with the rabbit anti-APP antibody, the next
incubation should be with a dilution of the first

15   detector reagent at a dilution determined empirically
based on its titer and affinity for the initial
antibody. Typically, the dilution of the first
detector reagent is about one to about six-fold less
than that of the second detector reagent. The second

20   detector is used to amplify the signal due to its
affinity for the first detection system. The increased
sensitivity appears to be related to the presence of
additional complexes that form first from a goat anti-
rabbit antibody followed by incubation with a rabbit

25   anti-goat antibody.

Those skilled in the art will appreciate that the
detector system can include any reagent or combination
of reagents suitable for detecting proteins in an
immunoblot format. Typically, an anti-antibody coupled

30   to a reporter is used as a detector reagent. Examples
of reporters include enzymes, such as, horseradish
peroxidase and alkaline phosphatase, radioisotopes,
chemiluminescent, fluorogenic or electrochemical
materials. Various coupling techniques are known to

35   those skilled in the art.

The following examples illustrate the invention:

Example 1
Production of APP-Specific Antibodies

Synthetic peptides, based on the amyloid precursor protein predicted amino acid sequence, were used as antigens to generate antibodies that could identify the entire APP. The peptides were synthesized by C. Brady (Du Pont) under the direction of J. Kauer using the RAMPS^{TM} method. These peptides corresponded substantially to amino acids numbered 599 to 618, 619 to 638, and 676 to 695 of the predicted amino acid sequence for APP (numbering was according to Kang group):

599 to 618:
GluPheArgHisAspSerGlyThrGluValHisHisGluLysLeuValPhePheAlaGlu

619 to 638:
AspValGlySerAsnLysGlyAlaIleIleGlyLeuMetValGlyGlyValValIleAla

676 to 695:
LysMetGlnGlnAsnGlyTyrGluAsnProThrTyrLysPhePheGluGlnMetGlnAsn

Three antisera, designated as numbers 382, 384 and 385, respectively, were generated. As detailed below, these antibodies proved useful in identifying the amyloid precursor.

The APP-specific antibodies (#382, 384, 385) were raised by repeated injection of each unconjugated peptide into separate female New Zealand albino rabbits. Initially, 0.5 mg peptide was injected at multiple subdermal sites in Freund's complete adjuvant. Subsequent booster injections of 0.2 mg peptide were performed at 3 to 4 week intervals using Freund's incomplete adjuvant. Rabbits were bled 7 to 14 days after the booster injections.

Serum titers were determined by using the following
enzyme-linked immunosorbent assay (ELISA): peptides
were bound to wells of polyvinyl microtiter plates (1
μg/well) which had been pre-treated with 0.1%

5   glutaraldehyde to facilitate peptide adhesion. Serial
dilutions of serum samples in bovine serum albumin
(hereinafter "BSA") BSA-phosphate buffered saline were
added to the wells which had been preblocked with BSA.
The samples were incubated for one hour. Bound

10   antibodies were detected by incubating the bound
antigen-antibody complex with goat anti-rabbit IgG
antibody conjugated to alkaline phosphatase which was
then reacted with paranitrophenylphosphate substrate
solution. Antisera yielding half-maximal signals in

15   the ELISA at dilutions of 1:500 or higher were stored
at -80°C.

Example 2
Immunohistochemical Analysis
Using APP-Specific Antibodies

20   Two of the antisera (#384 and #385) were subjected
to rigorous immunohistochemical analysis in order to
establish their specificity and determine the
localization of the precursor protein in the rat

25   central nervous system. The antisera were used in
combination with the avidin-biotin modification (Hsu et
al. J. Histochem. 29:577-580 (1981)) of the Sternberger
(In Immunocytochemistry, Colan and McClusky, eds.
(Wiley, NY) pp. 104-169 (1979)) immunoperoxidase

30   procedure, to localize the precursor protein at both
the light and electron microscopic level. The details
of these procedures have been published previously
(Card et al. Neuron 1:835-846 (1988)). Standard
procedures are described in Hsu et al. above and are

35   also described below. Sections of buffered aldehyde-

fixed tissue were incubated in various dilutions of
primary antisera for a period of 24 to 48 hours at 4°C,
followed by sequential exposure to a biotinylated
secondary antiserum and the avidin-biotin-peroxidase
solution. Diaminobenzidine and hydrogen peroxide were
then utilized to visualize antigen localization. Tests
of specificity included blocking of each antiserum with
its native antigen (i.e., blocking of antiserum #385
with the antigen used to generate the antiserum #385)
as well as cross blocking preabsorptions of each
antiserum with other peptide fragments. Antisera were
preincubated with 10 μM, 50 μM, and 100 μM
concentrations of antigen for 90 minutes at room
temperature prior to placing tissue sections in the
antiserum. Blocking each antiserum with its native
antigen eliminated all immunoreactivity, while cross
blocking preabsorptions did not compromise
immunohistochemical staining. Each test of specificity
was run in parallel to immunohistochemical localization
of the precursor protein with unblocked antisera to
verify the success of the immunohistochemical
procedure.

 Immunohistochemical localizations with the #384 and
#385 antisera revealed identical populations of cells
throughout the rodent neuraxis. Extensive, but
circumscribed, populations of immunoreactive neurons
were present in all areas of the CNS with the largest
concentration of cells occurring in the olfactory bulb,
the cerebral cortex, the basal forebrain and the
hippocampus. This localization of the precursor
protein in hippocampus and cortex was consistent with
studies in human brain which identified the amyloid
precursor mRNA in the same areas using in situ
hybridization. The cortex was also distinguished by a
small, but distinct, population of immunoreactive
astrocytes.

These studies were extended to human tissue in
cortex and hippocampus with all three antisera.
Antisera (including #382) were also used in
immunocytochemical analysis of human brain from normal
and Alzheimer's disease patients. Antiserum number 382
identified neuropathological plaques in both the cortex
and hippocampus of Alzheimer's patients. Antiserum
number 384 identifies both plaques and reactive
astroglia in cortex and hippocampus of Alzheimer's
brain. Antiserum number 385 did not prove useful for
immunocytochemical localizations in human brain.

Example 3
Immunoblot Analysis of
Human Cerebrospinal Fluid Samples

Cerebrospinal fluid (CSF) samples from patients
suffering from Alzheimer's disease (diagnosis confirmed
post-mortem) or Parkinson's disease or controls with no
history of neurological problems were obtained from the
National Neurological Research Bank (Los Angeles, CA).
Samples were concentrated by ultrafiltration over
Amicon YM-30 membranes. Protein concentrations were
determined by the method of Bradford (Anal. Biochem.
72:248,254 (1976)) and were equalized. Proteins in the
CSF samples were separated by SDS-polyacrylamide gel
electrophoresis on 5 to 15% linear gradient gels as
previously described (Siman et al. Proc. Natl. Acad.
Sci. USA 81:3572-3576 (1984)) and were transferred to
nitrocellulose filters by the Western blot technique
(Towbin et al. Proc. Natl. Acad. Sci. 76:4350:4354
(1979); Siman et al. J. Neurosci. 7:55-64 (1987)) and
blocked for 60 min with 5% dry milk in Tris-buffered
saline (TBS). Filters were then incubated for 2 hours
at 23°C with APP specific antibody #385 at 1:300

dilution in dry milk/TBS. After three 5 min washes
with TBS + 0.05% Tween-20 (TTBS), filters were
incubated for 1 hour with goat anti-rabbit IgG-alkaline
phosphatase (Bio-Rad) at 1:2000 dilution in dry

5   milk/TBS. After 3 TTBS washes, filters were incubated
a second time for another hour with rabbit anti-goat
IgG antibody coupled to alkaline phosphatase (Cappel)
at 1:500 dilution in dry milk/TBS. Filters were washed
twice with TTBS, twice with TBS and were developed in

10   the dark for 30 to 45 min with alkaline phosphatase
substrate solution consisting of BCIP/NBT (kit obtained
from Bio-Rad). Dried, stained blots were photographed
with Polaroid type 55 film. Antibodies #382 and #384
were also evaluated in this procedure.

15   CSF samples from 14 Alzheimer's disease patients, 9
Parkinson's disease patients, and 5 controls, and 13
others were examined by the immunoblot method.
Initially, 6 Alzheimer's samples and 5 controls were
concentrated by ultrafiltration over Amican YM-30

20   membranes to a total protein concentration of 1 mg/mL;
the remaining analysis was performed on samples
concentrated to 2.5 mg/mL. The optimal concentration
was determined empirically, i.e., it was desired to
shorten the time it took for signal to appear.

25   All 14 of the 14 Alzheimer's samples were found to
contain a polypeptide of apparent molecular size of
about 130 kDa that reacted with antibody #385 prepared
against the C-terminal portion of the β-amyloid
precursor protein (APP) (amino acids 676-695). Other

30   domains of APP can also be used to generate antibodies.
Among the Parkinson's disease and other samples, 9 of
22 samples contained the 130 kDa polypeptide in
detectable levels.

         Results discussed above are presented in Table 1.

35

TABLE 1[a]

SAMPLE NO.	AGE/SEX	DIAGNOSIS	130 kD	35 kD
7783	80/M	Alzheimer's	+	
7790	75/M	Alzheimer's	+	
9203	86/M	Alzheimer's	+	
9294	76/M	Alzheimer's	+	
9403	74/F	Alzheimer's	+	+
8637	74/F	Alzheimer's	+	+
8278	64/F	Alzheimer's	+	+
8119	68/F	Alzheimer's	+	+
9314	72/F	Alzheimer's	+	+
9277	69/M	Alzheimer's	+	+
9262	64/M	Alzheimer's	+	+
9368	70/F	Alzheimer's	+	+
9373	67/F	Alzheimer's	+	+
5116-A	67/M	Alzheimer's	+	+
3154	?/M	normal	-	-
2067	?/M	normal	-	-
3178	?/M	normal	-	-
3227	22/M	normal	-	-
3160	24/M	normal	-	-
8437	62/M	liver cirrhosis	+	
9131	70/M	heart failure	+	
9544	72/M	alcoholism/infarcts	-	
9945	86/M	pneumonia	+	
8360	68/M	Parkinson's	+	+
9821-0	64/M	Parkinson's	+	+
9800-0	66/F	Parkinson's	+	+
9674	77/M	Parkinson's	-	-
7891	66/M	Parkinson's	-	+
6166-A	76/M	Parkinson's	-	-
5407-A	75/M	Parkinson's	-	-
7749	75/M	Parkinson's	-	-
6068	50/M	Parkinson's	-	-
9424	85/M	acute hypoxia	+	+
9276	72/F	Creutzfeldt-Jakob	+	+
9124	76/M	Creutzfeldt-Jakob	-	-
9353	70/M	Pick's	+	+

[a]   A "+" indicates a positive response
       A "-" indicates a negative response and
       A blank indicates that the sample could not be
       analyzed.

Detection of an approximately 130 kDa polypeptide in the Alzheimer's samples required concentrating the samples to about 2.5 mg/mL and incubating the polypeptide-antibody complex once with the goat anti-rabbit IgG-alkaline phosphatase and then once with the rabbit anti-goat IgG-alkaline phosphatase. As was stated above, the amount to which the samples are concentrated depends to some extent on the sensitivity of the reporter system, the affinity of the antibodies as well as the quality of the samples. Thus, the 2.5 mg/ml figure will vary. Sensitivity of immunodetection increased about three-fold using the second incubation step. It is believed that this was due to presence of increased number of complexes as was noted above.

In 14 of 14 Alzheimer's samples, the 130 kDa polypeptide was consistently detected. The 130 kDa polypeptide was detected in only 9 of 22 non-Alzheimer's control samples.

The Alzheimer's samples and controls group did not differ significantly in their IgG or albumin content (data provided by National Neurological Research Bank) and Coomassie blue staining of SDS-polyacrylamide gels revealed no apparent differences in the level of content of any of the stained polypeptides.

These results are significant because they establish that a 130 kDa protein, in soluble form, was detected in a bodily fluid and appears to be an APP-related protein sharing at least one epitope with the C-terminus of APP corresponding substantially to amino acids 676 to 695 of APP or to a subsequence thereof.

The 130 kDa protein may be an intact APP isoform, as it co-migrates with the largest immunoreactive APP form present in rat brain. It may be an altered or homologous amyloid immunoreactive species.

   In addition to the 130 kDa polypeptide, an
immunopositive polypeptide of apparent molecular size
of about 35 kDa was elevated in the Alzheimer's disease
samples, being readily detectable in CSF of the ten

5   Alzheimer's samples analyzed. This band was not
detected in the normal controls. It was detected in 6
of 12 degenerative diseases (Parkinson's, Creutzfeldt-
Jakob or Pick's), and in 1 sample having unclear
neurological status (the other four samples were not

10 analyzable).
   The present results have established that a 35 kDa
protein, in soluble form, was also detected in a bodily
fluid and appears to share at least one epitope with
the C-terminus of APP corresponding substantially to

15 amino acids 676 to 695 of APP or to a subsequence
thereof.

20

25

30

35

<u>Claims</u>

1. A method to assist in the diagnosis of Alzheimer's
   disease comprising detecting, in bodily fluids, two
   APP-related proteins, in soluble form, said
   proteins have an apparent molecular size of about
   130 kDa and about 35 kDa, and each of said proteins
   shares at least one epitope with the C-terminus of
   APP corresponding substantially to amino acids
   676-695 of APP as shown in Figure 1.

2. A method according to claim 1 wherein the epitope
   corresponds substantially to a subsequence of amino
   acids 676-695 of APP as depicted in Figure 1.

3. A method according to claim 1 wherein the fluid is
   cerebrospinal fluid.

4. A method to assist in the diagnosis of Alzheimer's
   disease comprising detecting in bodily fluids two
   APP-related proteins, in soluble form, the proteins
   have an apparent molecular size of about 130 kDa
   and about 35 kDa, and each of the proteins shares
   at least one epitope with the C-terminus of APP
   corresponding substantially to amino acids 676-695
   of APP as shown in Figure 1 wherein the proteins
   are concentrated from bodily fluids, said
   concentrated proteins are reacted in an immunoblot
   format with at least one antibody specific for the
   C-terminus of APP corresponding substantially to
   amino acids 676-695 of APP as shown in Figure 1 and
   the product of the reaction is detected.

5. A method according to claim 4 wherein the epitope
    corresponds substantially to a subsequence of amino
    acids 676-695 of APP as shown in Figure 1 and the
    antibody is specific for a subsequence of amino
    acids 676-695 of APP as shown in Figure 1.

6. A method according to claim 4 wherein the fluid is
    cerebrospinal fluid.

7. A method according to claim 4 wherein the proteins
    are concentrated to a range from about 1.0 mg/ml to
    about 3.5 mg/ml.

8. A method according to claim 4 wherein the reaction
    product is detected by incubating with a first
    detector reagent which is specific for the antibody
    directed to the C-terminus of APP followed by
    reaction with a second detector reagent which is
    specific for the first detector reagent and which
    enhances detection of the two APP-related proteins.

9. A method according to claim 4 wherein the proteins
    are concentrated from bodily fluids by
    ultrafiltration.

10. A method according to claim 4 wherein the detector
    reagents are anti-antibodies, each of which is
    coupled to a reporter selected from the group
    consisting of enzymes, radioisotopes,
    chemiluminescent, fluorogenic and electrochemical
    materials.

Figure 1

Nucleotide sequence for the APP cDNA and encoded
of the amino acid sequence gene product
according to Kang et al.:

```
      ATGCTGCCCGGTTTGGCACTGCTCCTGCTGGCCGCCTGGACGGCTCGGGCGCTGGAGGTA
147   ---+----------+----------+----------+----------+----------+------ 206
      TACGACGGGCCAAACCGTGACGAGGACGACCGGCGGACCTGCCGAGCCCGCGACCTCCAT

c     M  L  P  G  L  A  L  L  L  L  A  A  W  T  A  R  A  L  E  V    -

      CCCACTGATGGTAATGCTGGCCTGCTGGCTGAACCCCAGATTGCCATGTTCTGTGGCAGA
207   ---+----------+----------+----------+----------+----------+------ 266
      GGGTGACTACCATTACGACCGGACGACCGACTTGGGGTCTAACGGTACAAGACACCGTCT

c     P  T  D  G  N  A  G  L  L  A  E  P  Q  I  A  M  F  C  G  R    -

      CTGAACATGCACATGAATGTCCAGAATGGGAAGTGGGATTCAGATCCATCAGGGACCAAA
267   ---+----------+----------+----------+----------+----------+------ 326
      GACTTGTACGTGTACTTACAGGTCTTACCCTTCACCCTAAGTCTAGGTAGTCCCTGGTTT

c     L  N  M  H  M  N  V  Q  N  G  K  W  D  S  D  P  S  G  T  K    -

      ACCTGCATTGATACCAAGGAAGGCATCCTGCAGTATTGCCAAGAAGTCTACCCTGAACTG
327   ---+----------+----------+----------+----------+----------+------ 386
      TGGACGTAACTATGGTTCCTTCCGTAGGACGTCATAACGGTTCTTCAGATGGGACTTGAC

c     T  C  I  D  T  K  E  G  I  L  Q  Y  C  Q  E  V  Y  P  E  L    -

      CAGATCACCAATGTGGTAGAAGCCAACCAACCAGTGACCATCCAGAACTGGTGCAAGCGG
387   ---+----------+----------+----------+----------+----------+------ 446
      GTCTAGTGGTTACACCATCTTCGGTTGGTTGGTCACTGGTAGGTCTTGACCACGTTCGCC

c     Q  I  T  N  V  V  E  A  N  Q  P  V  T  I  Q  N  W  C  K·R    -

      GGCCGCAAGCAGTGCAAGACCCATCCCCACTTTGTGATTCCCTACCGCTGCTTAGTTGGT
447   ---+----------+----------+----------+----------+----------+------ 506
      CCGGCGTTCGTCACGTTCTGGGTAGGGGTGAAACACTAAGGGATGGCGACGAATCAACCA

c     G  R  K  Q  C  K  T  H  P  H  F  V  I  P  Y  R  C  L·V  G    -
```

2

```
     GAGTTTGTAAGTGATGCCCTTCTCGTTCCTGACAAGTGCAAATTCTTACACCAGGAGAGG
507  ---+---------+---------+---------+---------+---------+------ 566
     CTCAAACATTCACTACGGGAAGAGCAAGGACTGTTCACGTTTAAGAATGTGGTCCTCTCC

   c  E  F  V  S  D  A  L  L  V  P  D  K  C  K  F  L  H  Q  E  R   -

     ATGGATGTTTGCGAAACTCATCTTCACTGGCACACCGTCGCCAAAGAGACATGCAGTGAG
567  ---+---------+---------+---------+---------+---------+------ 626
     TACCTACAAACGCTTTGAGTAGAAGTGACCGTGTGGCAGCGGTTTCTCTGTACGTCACTC

   c  M  D  V  C  E  T  H  L  H  W  H  T  V  A  K  E  T  C  S  E   -

     AAGAGTACCAACTTGCATGACTACGGCATGTTGCTGCCCTGCGGAATTGACAAGTTCCGA
627  ---+---------+---------+---------+---------+---------+------ 686
     TTCTCATGGTTGAACGTACTGATGCCGTACAACGACGGGACGCCTTAACTGTTCAAGGCT

   c  K  S  T  N  L  H  D  Y  G  M  L  L  P  C  G  I  D  K  F  R   -

     GGGGTAGAGTTTGTGTGTTGCCCACTGGCTGAAGAAAGTGACAATGTGGATTCTGCTGAT
687  ---+---------+---------+---------+---------+---------+------ 746
     CCCCATCTCAAACACACAACGGGTGACCGACTTCTTTCACTGTTACACCTAAGACGACTA

   c  G  V  E  F  V  C  C  P  L  A  E  E  S  D  N  V  D  S  A  D   -

     GCGGAGGAGGATGACTCGGATGTCTGGTGGGGCGGAGCAGACACAGACTATGCAGATGGG
747  ---+---------+---------+---------+---------+---------+------ 806
     CGCCTCCTCCTACTGAGCCTACAGACCACCCCGCCTCGTCTGTGTCTGATACGTCTACCC

   c  A  E  E  D  D  S  D  V  W  W  G  G  A  D  T  D  Y  A  D  G   -

     AGTGAAGACAAAGTAGTAGAAGTAGCAGAGGAGGAAGAAGTGGCTGAGGTGGAAGAAGAA
807  ---+---------+---------+---------+---------+---------+------ 866
     TCACTTCTGTTTCATCATCTTCATCGTCTCCTCCTTCTTCACCGACTCCACCTTCTTCTT

   c  S  E  D  K  V  V  E  V  A  E  E  E  V  A  E  V  E  E  E   -

     GAAGCCGATGATGACGAGGACGATGAGGATGGTGATGAGGTAGAGGAAGAGGCTGAGGAA
867  ---+---------+---------+---------+---------+---------+------ 926
     CTTCGGCTACTACTGCTCCTGCTACTCCTACCACTACTCCATCTCCTTCTCCGACTCCTT

   c  E  A  D  D  D  E  D  D  E  D  G  D  E  V  E  E  E  A  E  E   -

     CCCTACGAAGAAGCCACAGAGAGAACCACCAGCATTGCCACCACCACCACCACCACCACA
927  ---+---------+---------+---------+---------+---------+------ 986
     GGGATGCTTCTTCGGTGTCTCTCTTGGTGGTCGTAACGGTGGTGGTGGTGGTGGTGGTGT

   c  P  Y  E  E  A  T  E  R  T  T  S  I  A  T  T  T  T  T  T  T   -
```

```
          GAGTCTGTGGAAGAGGTGGTTCGAG*TTCCTACAACAGCAGCCAGTACCCTGATGCCGTT
      987 ---+---------+---------+---------+---------+---------+------ 1046
          CTCAGACACCTTCTCCACCAAGCTCAAGGATGTTGTCGTCGGTCATGGGGACTACGGCAA

        C  E  S  V  E  E  V  V  R  V  P  T  T  A  A  S  T  P  D  A  V  -

          GACAAGTATCTCGAGACACCTGGGGATGAGAATGAACATGCCCATTTCCAGAAAGCCAAA
     1047 ---+---------+---------+---------+---------+---------+------ 1106
          TGTTCATAGAGCTCTGTGGACCCCTACTCTTACTTGTACGGGTAAAGGTCTTTCGGTTT

        C  D  K  Y  L  E  T  P  G  D  E  N  E  H  A  H  F  Q  K  A  K  -

          GAGAGGCTTGAGGCCAAGCACCGAGAGAGAATGTCCCAGGTCATGAGAGAATGGGAAGAG
     1107 ---+---------+---------+---------+---------+---------+------ 1166
          CTCTCCGAACTCCGGTTCGTGGCTCTCTCTTACAGGGTCCAGTACTCTCTTACCCTTCTC

        C  E  R  L  E  A  K  H  R  E  R  M  S  Q  V  M  R  E  W  E  E  -

          GCAGAACGTCAAGCAAAGAACTTGCCTAAAGCTGATAAGAAGGCAGTTATCCAGCATTTC
     1167 ---+---------+---------+---------+---------+---------+------ 1226
          CGTCTTGCAGTTCGTTTCTTGAACGGATTTCGACTATTCTTCCGTCAATAGGTCGTAAAG

        C  A  E  R  Q  A  K  N  L  P  K  A  D  K  K  A  V  I  Q  H  F  -

          CAGGAGAAAGTGGAATCTTTGGAACAGGAAGCAGCCAACGAGAGACAGCAGCTGGTGGAG
     1227 ---+---------+---------+---------+---------+---------+------ 1286
          GTCCTCTTTCACCTTAGAAACCTTGTCCTTCGTCGGTTGCTCTCTGTCGTCGACCACCTC

        C  Q  E  K  V  E  S  L  E  Q  E  A  A  N  E  R  Q  Q  L  V  E  -

          ACACACATGGCCAGAGTGGAAGCCATGCTCAATGACCGCCGCCGCCTGGCCCTGGAGAAC
     1287 ---+---------+---------+---------+---------+---------+------ 1346
          TGTGTGTACCGGTCTCACCTTCGGTACGAGTTACTGGCGGCGGCGGACCGGGACCTCTTG

        C  T  H  M  A  R  V  E  A  M  L  N  D  R  R  R  L  A  L  E  N  -

          TACATCACCGCTCTGCAGGCTGTTCCTCCTCGGCCTCGTCACGTGTTCAATATGCTAAAG
     1347 ---+---------+---------+---------+---------+---------+------ 1406
          ATGTAGTGGCGAGACGTCCGACAAGGAGGAGCCGGAGCAGTGCACAAGTTATACGATTTC

        C  Y  I  T  A  L  Q  A  V  P  P  R  P  R  H  V  F  N  M  L  K  -

          AAGTATGTCCGCGCAGAACAGAAGGACAGACAGCACACCCTAAAGCATTTCGAGCATGTG
     1407 ---+---------+---------+---------+---------+---------+------ 1466
          TTCATACAGGCGCGTCTTGTCTTCCTGTCTGTCGTGTGGGATTTCGTAAAGCTCGTACAC

        C  K  Y  V  R  A  E  Q  K  D  R  Q  H  T  L  K  H  F  E  H  V  -
```

*Different exon utilization results in the insertion of
additional amino acid sequences here leading to at least two
alternate APP forms; other alternate forms of the APP gene
expression may also exist.

4

```
      CGCATGGTGGATCCCAAGAAAGCCGCTCAGATCCGGTCCCAGGTTATGACACACCTCCGT
1467  ---+---------+---------+---------+---------+---------+------ 1526
      GCGTACCACCTAGGGTTCTTTCGGCGAGTCTAGGCCAGGGTCCAATACTGTGTGGAGGCA

   c   R  M  V  D  P  K  K  A  A  Q  I  R  S  Q  V  M  T  H  L  R   -

      GTGATTTATGAGCGCATGAATCAGTCTCTCTCCCTGCTCTACAACGTGCCTGCAGTGGCC
1527  ---+---------+---------+---------+---------+---------+------ 1586
      CACTAAATACTCGCGTACTTAGTCAGAGAGAGGGACGAGATGTTGCACGGACGTCACCGG

   c   V  I  Y  E  R  M  N  Q  S  L  S  L  L  Y  N  V  P  A  V  A   -

      GAGGAGATTCAGGATGAAGTTGATGAGCTGCTTCAGAAAGAGCAAAACTATTCAGATGAC
1587  ---+---------+---------+---------+---------+---------+------ 1646
      CTCCTCTAAGTCCTACTTCAACTACTCGACGAAGTCTTTCTCGTTTTGATAAGTCTACTG

   c   E  E  I  Q  D  E  V  D  E  L  L  Q  K  E  Q  N  Y  S  D  D   -

      GTCTTGGCCAACATGATTAGTGAACCAAGGATCAGTTACGGAAACGATGCTCTCATGCCA
1647  ---+---------+---------+---------+---------+---------+------ 1706
      CAGAACCGGTTGTACTAATCACTTGGTTCCTAGTCAATGCCTTTGCTACGAGAGTACGGT

   c   V  L  A  N  M  I  S  E  P  R  I  S  Y  G  N  D  A  L  M  P   -

      TCTTTGACCGAAACGAAAACCACCGTGGAGCTCCTTCCCGTGATTGGAGAGTTCAGCCTG
1707  ---+---------+---------+---------+---------+---------+------ 1766
      AGAAACTGGCTTTGCTTTTGGTGGCACCTCGAGGAAGGGCACTAACCTCTCAAGTCGGAC

   c   S  L  T  E  T  K  T  T  V  E  L  L  P  V  I  G  E  F  S  L   -

      GACGATCTCCAGCCGTGGCATTCTTTTGGGGCTGACTCTGTGCCAGCCAACACAGAAAAC
1767  ---+---------+---------+---------+---------+---------+------ 1826
      CTGCTAGAGGTCGGCACCGTAAGAAAACCCCGACTGAGACACGGTCGGTTGTGTCTTTTG

   c   D  D  L  Q  P  W  H  S  F  G  A  D  S  V  P  A  N  T  E  N   -

      GAAGTTGAGCCTGTTGATGCCCGCCCTGCTGCCGACCGAGGACTGACCACTCGACCAGGT
1827  ---+---------+---------+---------+---------+---------+------ 1886
      CTTCAACTCGGACAACTACGGGCGGGACGACGGCTGGCTCCTGACTGGTGAGCTGGTCCA

   c   E  V  E  P  V  D  A  R  P  A  A  D  R  G  L  T  T  R  P  G   -

      TCTGGGTTGACAAATATCAAGACGGAGGAGATCTCTGAAGTGAAGATGGATGCAGAATTC
1887  ---+---------+---------+---------+---------+---------+------ 1946
      AGACCCAACTGTTTATAGTTCTGCCTCCTCTAGAGACTTCACTTCTACCTACGTCTTAAG

   c   S  G  L  T  N  I  K  T  E  E  I  S  E  V  K  M  D  A  E  F   -
```

5

```
      CGACATGACTCAGGATATGAAGTTCATCATCAAAAATTGGTGTTCTTTGCAGAAGATGTG
 1947 ---+---------+---------+---------+---------+---------+------ 2006
      GCTGTACTGAGTCCTATACTTCAAGTAGTAGTTTTTAACCACAAGAAACGTCTTCTACAC

 c    R  H  D  S  G  Y  E  V  H  H  Q  K  L  V  F  F  A  E  D  V  -

      GGTTCAAACAAAGGTGCAATCATTGGACTCATGGTGGGCGGTGTTGTCATAGCGACAGTG
 2007 ---+---------+---------+---------+---------+---------+------ 2066
      CCAAGTTTGTTTCCACGTTAGTAACCTGAGTACCACCCGCCACAACAGTATCGCTGTCAC

 c    G  S  N  K  G  A  I  I  G  L  M  V  G  G  V  V  I  A  T  V  -

      ATCGTCATCACCTTGGTGATGCTGAAGAAGAAACAGTACACATCCATTCATCATGGTGTG
 2067 ---+---------+---------+---------+---------+---------+------ 2126
      TAGCAGTAGTGGAACCACTACGACTTCTTCTTTGTCATGTGTAGGTAAGTAGTACCACAC

 c    I  V  I  T  L  V  M  L  K  K  K  Q  Y  T  S  I  H  H  G  V  -

      GTGGAGGTTGACGCCGCTGTCACCCCAGAGGAGCGCCACCTGTCCAAGATGCAGCAGAAC
 2127 ---+---------+---------+---------+---------+---------+------ 2186
      CACCTCCAACTGCGGCGACAGTGGGGTCTCCTCGCGGTGGACAGGTTCTACGTCGTCTTG

 c    V  E  V  D  A  A  V  T  P  E  E  R  H  L  S  <u>K  M  Q  Q  N</u>  -

      GGCTACGAAAATCCAACCTACAAGTTCTTTGAGCAGATGCAGAACTAG
 2187 ---+---------+---------+---------+---------+---- 2234
      CCGATGCTTTTAGGTTGGATGTTCAAGAAACTCGTCTACGTCTTGATC

 c    <u>G  Y  E  N  P  T  Y  K  F  F  E  Q  M  Q  N</u>  *  -
```

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) °

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁵: G 01 N 33/68

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
IPC⁵	G 01 N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included In the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category °	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	Proc. Natl. Acad. Sci. USA, vol. 85, October 1988, D.J. Selkoe et al.: "Beta-amyloid precursor protein of Alzheimer disease occurs as 110- to 135-kilo-dalton membrane-associated proteins in neural and nonneural tissues", pages 7341-7345 see the whole article cited in the application --	1,2,4,5
A	EP, A, 0274826 (CALIFORNIA BIOTECHNOLOGY INC.) 20 July 1988 see page 1, line 1 - page 5, line 55; page 9, line 50 - page 10, line 19 cited in the application --	1-10
	,	./.

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
6th September 1990	2 7 SEP 1990

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	Mme N. KUIPER

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, '' with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A,P	Proc. Natl. Acad. Sci. USA, vol. 87, March 1990, H. Arai et al.: "Defined neurofilament, tau, and beta-amyloid precursor protein epitopes distinguish Alzheimer from non-Alzheimer senile plaques", pages 2249-2253 see the whole article --	1-6
A,P	Experimental Neurology, vol. 106, no. 3, September 1989, Academic Press, Inc., L.I. Benowitz et al.: "The amyloid precursor protein is concentrated in neuronal lysosomes in normal and Alzheimer disease subjects", pages 237-250 see the whole article --	1,2,4,5
A,P	Biochemical and Biophysical Research Communications, vol. 163, no. 1, 30 August 1989, Academic Press, Inc., J. Ghiso et al.: "Alzheimer's disease amyloid precursor protein is present in senile plaques and cerebrospinal fluid", pages 430-437 see the whole article ----------	1-6

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0274826	20-07-88	AU-A-	8329087	16-06-88
		EP-A-	0332640	20-09-89
		WO-A-	8803951	02-06-88

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82